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A wortmannin-sensitive phosphatidylinositol 4-kinase that regulates hormone-sensitive pools of inositolphospholipids

SATOSHI NAKANISHI*, KEVIN J. CATT, AND TAMAS BALLA†

Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, National Institutes of Health, Building 49, Room 6A 36, 9000 Rockville Pike, Bethesda, MD 20892

Communicated by Henry Metzger, National Institute of Arthritis and Musculoskeletal and Skin Diseases, Bethesda, MD, February 9, 1995 (received for review December 27, 1994)

ABSTRACT **The synthesis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2], the immediate precursor of intracellular signals generated by calcium-mobilizing hormones and growth factors, is initiated by the conversion of phosphatidylinositol to phosphatidylinositol 4-phosphate [PtdIns(4)P] by phosphatidylinositol 4-kinase (PtdIns 4-kinase). Although cells contain several PtdIns 4-kinases, the enzyme responsible for regulating the synthesis of hormone-sensitive PtdIns(4,5)P_2 pools has not been identified. In this report we describe the inhibitory effect of micromolar concentrations of wortmannin (WT) on the synthesis of hormone-sensitive PtdIns(4)P and PtdIns(4,5)P_2 pools in intact adrenal glomerulosa cells, and the presence of a WT- sensitive PtdIns 4-kinase in adrenocortical extracts. In addition to its sensitivity to the PtdIns 3-kinase inhibitor WT, this enzyme is distinguished from the recognized membrane-bound PtdIns 4-kinases by its molecular size and weak membrane association. Inhibition of this PtdIns 4-kinase by WT results in rapid loss of the hormone-sensitive PtdIns(4,5)P_2 pool in angiotensin II-stimulated glomerulosa cells. Consequently, WT treatment inhibits the sustained but not the initial increases in inositol 1,4,5-trisphosphate and cytoplasmic [Ca^{2+}] in a variety of agonist-stimulated cells, including adrenal glomerulosa cells, NIH 3T3 fibroblasts, and Jurkat lymphoblasts. These results indicate that a specific WT-sensitive PtdIns 4-kinase is critical for the maintenance of the agonist-sensitive polyphosphoinositide pool in several cell types.**

Phosphatidylinositol 4,5-phosphate [PtdIns(4,5)P_2] is a critical plasma-membrane precursor of several of the intracellular messengers that mediate the actions of many hormones, transmitters, and growth factors (2–4). PtdIns(4,5)P_2 has also been implicated in other cellular functions, including regulation of the activity of certain actin-binding proteins (5) and of clathrin assembly and, hence, receptor endocytosis (6). Hormone-sensitive and -insensitive pools of inositolphospholipids have been described (7–9), as well as a nuclear inositolphospholipid system that appears to be regulated independently of receptor-activated inositolphospholipid turnover (10). The production of polyphosphoinositides from phosphatidylinositol (PtdIns) is therefore of vital importance for multiple aspects of cell regulation.

The first step in the production of PtdIns(4,5)P_2 is catalyzed by PtdIns 4-kinases, several forms of which have been isolated from mammalian sources as well as from yeast (11–13). Two membrane-bound mammalian PtdIns 4-kinases have been characterized, a 56-kDa type II enzyme that is inhibited by adenosine and an ≈200-kDa type III enzyme that is relatively insensitive to adenosine. The mammalian type I enzyme, which phosphorylates PtdIns at the 3 position, is largely cytoplasmic in resting cells and contains 110-kDa catalytic and 85-kDa regulatory subunits (14, 15). The catalytic domain has significant homology with the *Saccharomyces cerevisiae* PtdIns 3-kinase Vps34p (16) and with a recently cloned yeast cytoplasmic PtdIns 4-kinase (13). The latter enzyme, which has a molecular mass of 125 kDa, was isolated from the cytosolic fraction and appears distinct from the 45- to 55-kDa enzyme isolated from yeast membranes (17). The soluble enzyme was found to be essential for yeast viability, and the 45- to 55-kDa membrane-bound enzyme (if distinct from the former) cannot substitute for its function. On the basis of sequence homologies within the catalytic domain of these enzymes, additional yeast enzymes (TOR1 and TOR2) (18) and a recently cloned human protein (RAFT1) (19) have been identified as PtdIns kinase homologs, the latter two also being targets of the immunophilin drug rapamycin.

This heterogeneity of the known PtdIns 4-kinases raises the questions of which (if any) of these enzymes controls the formation of the agonist-sensitive inositolphospholipids required for intracellular signaling. In the present study we describe the properties of a PtdIns 4-kinase identified in the adrenal cortex. In contrast to the known tightly membrane-bound PtdIns 4-kinases, this loosely membrane-associated enzyme is sensitive to the potent PtdIn 3-kinase inhibitor wortmannin (WT) and appears to be responsible for maintenance of the hormone-sensitive pools of inositolphospholipids in several cell types.

MATERIAL AND METHODS

Cell Culture. Adrenal glomerulosa cells were prepared from bovine adrenal cortical tissue and cultured as described (20). The NIH 3T3 cells used were from previously characterized clones of cells stably transfected with the pZip/Neo plasmid (21). The Jurkat lymphoblastoma cell line E61 was provided by Richard Youle (National Institutes of Neurological Disorders and Strokes, Bethesda, MD). NIH 3T3 cells were cultured in DMEM-high glucose medium/L-glutamine/10% fetal bovine serum, and Jurkat cells were maintained in RPMI 1640 medium/L-glutamine/50 μM 2-mercaptoethanol/10% fetal bovine serum.

Measurements of Inositol Phosphate Formation. Procedures for the determination of inositol phosphate levels in [^{3}H]inositol-labeled glomerulosa cells and NIH 3T3 cells have been described elsewhere (21, 22). Jurkat cells were labeled in modified DMEM-high glucose medium/0.2 μM *myo*-inositol/L-glutamine/0.1% bovine serum albumin/5% serum containing *myo*-[^{3}H]inositol (10 μCi/ml; 1 Ci = 37 GBq) at a density of 5–8 × 10^6 cells per ml for 24 h. After two washes with

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Abbreviations: Ang-II, angiotensin II; WT, wortmannin; [Ca^{2+}]$_i$, cytoplasmic Ca^{2+} concentration; PtdIns, phosphatidylinositol. Inositol lipids and phosphates are abbreviated according to the International Union of Pure and Applied Biochemistry nomenclature (1).

*Present address: Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., 3-6-6 Asahimachi, Machida-shi, Tokyo 194, Japan.
†To whom reprint requests should be addressed.

DMEM-high glucose medium/L-glutamine/0.1% bovine serum albumin/10 mM Hepes (pH 7.4), cells were resuspended in the same medium at a density of 4×10^7 cells per ml and aliquoted at 200 μl into polystyrene tubes. After a 10-min preincubation at 37°C, WT (10 μM final) or dimethyl sulfoxide was added, and cells were incubated for an additional 10 min. Cells were then stimulated with antibody against CD3 at 10 μg/ml (OKT-3), and incubations were continued for selected times. Reactions were terminated by addition of 200 μl of ice-cold perchloric acid. Inositol phosphates were extracted and separated on HPLC using a Synchropak column (Thomson Instruments, Vienna, VA) as described (21).

Measurements of Cytoplasmic Ca^{2+} Concentration. Measurements of $[Ca^{2+}]_i$ were done in fura-2-loaded glomerulosa cells and NIH 3T3 cells, as described (21, 23). Jurkat cells were loaded with 0.5 μM fura-2/AM for 1 hr at room temperature, at a density of 4×10^6 cells per ml. After being washed, cells were kept in DMEM-high glucose/L-glutamine/0.1% bovine serum albumin at room temperature, and $5–6 \times 10^6$ cells were spun immediately before the $[Ca^{2+}]_i$ measurement. Cells were incubated at 34°C in 2.5 ml of modified Krebs–Ringer solution (1.2 mM $CaCl_2$/2.42 mM KCl/1.18 mM KH_2PO_4/118 mM NaCl/0.8 mM $MgSO_4$/5 mM $NaHCO_3$/20 mM Hepes·NaOH, pH 7.4/10 mM glucose). Fluorescence emission of fura-2 was monitored by a Deltascan dual wavelength fluorescence photometer (Photon Technology International, Princeton, NJ).

Analysis of ^{32}P- and ^{3}H-labeled Inositolphospholipids in Adrenal Glomerulosa Cells. Glomerulosa cells were cultured on 4-well culture plates (Nunc) and labeled with *o*-[^{32}P]phosphate (2 μCi/ml) for 4 hr in 0.2 ml of a low-phosphate Krebs–Ringer solution (118 mM NaCl/1.2 mM $CaCl_2$/3.6 mM KCl/0.8 mM $MgSO_4$/22.4 μM KH_2PO_4/5 mM $NaHCO_3$/20 mM Hepes·NaOH, pH 7.4/10 mM glucose) containing 0.1% bovine serum albumin. Inhibitors were added 10 min before stimulation with angiotensin II (Ang-II) for the indicated times, and reactions were stopped by the addition of ice-cold perchloric acid (5% final). Lipids were extracted from the cell pellets and separated on preimpregnated TLC plates, as described (22). For quantitation of the labeled lipids, radioactive spots were identified by autoradiography (7–9 days at −70°C for ^{3}H-labeled samples) and cut out for radioactivity measurements in a liquid scintillation counter. In the case of ^{32}P-labeled samples, TLC plates were also analyzed with a PhosphorImager (Molecular Dynamics) using standards of known ^{32}P activity to determine the linear range and to convert density readings to radioactivities.

Preparation of PtdIns 4-Kinase and PtdIns(4)*P* 5-Kinase. The cortical tissue from five bovine adrenal glands was homogenized in ice-cold buffer A (20 mM Tris·HCl, pH 7.4/1 mM EDTA/1 mM dithiothreitol/0.1 mM phenylmethylsulfonyl fluoride/leupeptin at 10 μg/ml with a Teflon homogenizer and centrifuged at 100,000 × *g* for 60 min. The supernatant (S1) was removed, and the pellet was homogenized in the same buffer and centrifuged at 100,000 × *g* for 60 min. The supernatant (S2) was removed again, and the pellet was resuspended in buffer A/1 M NaCl. The suspension was stirred for 30 min at 4°C and centrifuged at 100,000 × *g* for 60 min. The resulting supernatant (S3) was removed, and the pellet (P3) was resuspended in buffer containing 1 M NaCl. PtdIns 4-kinase activity was recovered in the S3 and P3 fractions, and the PtdIns(4)*P* 5-kinase activity was recovered in the S2 fraction. For separation of WT-sensitive and -insensitive soluble PtdIns 4-kinase activity, the S3 fraction was subjected to $(NH_4)_2SO_4$ precipitation and subsequent DEAE-Sepharose column chromatography. The protein fraction precipitated with $(NH_4)_2SO_4$ (22.6 g/100 ml) was collected by centrifugation for 20 min at 10,000 × *g* and dissolved in buffer A. After dialysis against the same buffer, the protein solution was applied to a DEAE-Sepharose fast-flow column (1.6 cm × 8 cm) preequilibrated with buffer A. The column was washed with 7.5 ml of buffer A, and proteins were eluted with a linear gradient of 0–0.5 M NaCl in buffer A (100 ml of total volume). Fractions were assayed for PtdIns 4-kinase activity, and those containing WT-sensitive activity were pooled, dialyzed, concentrated, and stored at −80°C for at least 2 weeks without loss of activity.

Assay of PtdIns 4-Kinase and PtdIns(4)*P* 5-Kinase Activities. The activities of PtdIns 4-kinase and PtdIns(4)*P* 5-kinase were measured by incorporation of radioactivity from [γ-^{32}P]ATP into organic solvent-extractable material (24, 25). The standard reaction mixture for PtdIns 4-kinase (50 μl final volume) contained 50 mM Tris·HCl (pH 7.5), 20 mM $MgCl_2$, 1 mM EGTA, 1 mM PtdIns, 0.4% Triton X-100, bovine serum albumin at 0.5 mg/ml, 100 μM [γ-^{32}P]ATP, and the enzyme. The reaction mixture for PtdIns(4)*P* 5-kinase (50-μl final volume) contained 0.25 M sucrose, 50 mM Tris·HCl (pH 7.5), 15 mM $MgCl_2$, 1 mM EGTA, 80 μM PtdIns(4)*P*, 80 μM phosphatidylserine, 0.04% Triton X-100, bovine serum albumin at 0.5 mg/ml, 0.1% PEG 20,000, 100 μM [γ-^{32}P]ATP, and the enzyme. All assay components except [γ-^{32}P]ATP were preincubated with inhibitors for 5 min at 30°C. Reactions were started by addition of [γ-^{32}P]ATP, incubated for 8 min, and terminated by the addition of 3 ml of $CHCl_3/CH_3OH$/concentrated HCl, 200:100:0.75 (vol/vol). The organic solvent phase was separated from [γ-^{32}P]ATP by adding 0.6 ml of 0.6 M HCl, mixing vigorously, and left standing for phase separation. The upper (aqueous) phase was discarded, and 1.5 ml of $CHCl_3$:CH_3OH:0.6 M HCl, 3:48:47 (vol/vol) was added to the lower phase, followed by mixing and phase separation. The lower phase was then transferred to scintillation vials, and after evaporation the radioactivity was analyzed by liquid scintillation spectrometry. The enzyme concentrations were chosen so that the reactions of both PtdIns 4-kinase and PtdIns(4)*P* 5-kinase were linear during the 8-min incubation. More than 98% of the radioactive products in the reaction mixtures of PtdIns 4-kinase and PtdIns(4)*P* 5-kinase assays comigrated with PtdIns(4)*P* and PtdIns(4,5)P_2 standards, respectively, as assessed after TLC separation (22). The PtdIns*P* product of the PtdIns-kinase assay was also identified by resolving the ^{32}P-labeled products in a TLC-system that clearly separates PtdIns(3)*P* and PtdIns(4)*P* (26).

PtdIns 3-Kinase Measurements. Cultured bovine glomerulosa cells (≈10^8 cells) were removed from the culture plates with versene and after centrifugation were resuspended in 3 ml of ice-cold lysis buffer (20 mM Tris·HCl, pH 7.5/150 mM NaCl/5 mM EGTA/1% Nonidet P-40/1 mM Na_3VO_4/1 mM phenylmethylsulfonyl fluoride). Anti-p85 antibody (10–20 μl) was then added, and the mixture was kept on ice for 2 hr before adding protein A–Sepharose for an additional hour. The beads were then washed three times with lysis buffer and then with LiCl buffer (0.5 M LiCl/0.1 M Tris·HCl, pH 7.5/1 mM phenylmethylsulfonyl fluoride) and finally with a wash buffer (0.1 M NaCl/1 mM EDTA/20 mM Tris·HCl, pH 7.5/1 mM phenylmethylsulfonyl fluoride). The assay was done in a 50-μl reaction mixture containing PtdIns (0.08 mg/ml), bovine serum albumin (0.5 mg/ml), 20 mM Tris·HCl (pH 7.4), 1 mM EGTA, 20 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP (1 μCi per tube), and the immunoprecipitated enzyme. Reactions were performed at 30°C for 30 min. The lipids were then extracted and quantitated as described for the PtdIns 4-kinase assay.

Materials. *myo*-[^{3}H]Inositol (80–120 Ci/mmol), K_2H[^{32}P]-PO_4 (200 mCi/mmol), and [γ-^{32}P]ATP (0.5–3 Ci/mmol) were purchased from Amersham. Fura-2 acetoxymethyl ester was from Molecular Probes. Adenosine, 5′-chloro-5′-deoxyadenosine, PtdIns, and phosphatidylserine were from Sigma. PtdIns(4) and PtdIns(4,5)P_2 were from Boehringer Mannheim. DEAE–Sepharose fast flow and protein A–Sepharose were from Pharmacia. The anti-p85 antibody for PtdIns 3-kinase was purchased from Upstate Biotechnology. Culture media were ob-

tained from Biofluids (Rockville, MD) and the National Institutes of Health media unit. WT was a gift of Tokyo Research Laboratories, Kyowa Hakko Kogyo, Tokyo. All other reagents were of analytical or HPLC grade.

RESULTS AND DISCUSSION

The importance of the soluble yeast PtdIns 4-kinase in cell viability and its structural similarity to the PtdIns 3-kinases raised the question of whether soluble PtdIns 4-kinase(s) could also be recovered from mammalian tissues, in which only the membrane-bound enzymes have been studied. As expected, most of the PtdIns 4-kinase activity of bovine adrenocortical homogenate was associated with the membrane fraction. However, a small proportion (≈20%) of this activity could be extracted from the membranes with 1 M NaCl, and ≈50% of this soluble activity was inhibited by submicromolar concentrations of the PtdIns 3-kinase inhibitor WT. In contrast, the tightly membrane-bound PtdIns 4-kinase(s), as well as the soluble PtdIns(4)*P* 5-kinase, were insensitive to WT (Fig. 1*A*). The WT-sensitive and -insensitive soluble PtdIns 4-kinases could be separated on DEAE–Sepharose, which retained the WT-sensitive enzyme, whereas the WT-insensitive activity was eluted at low ionic strength (Fig. 1*B*). The PtdIns 4-kinase activity eluted from the column with 0.25 M NaCl was completely inhibited by 10 μM WT, with an IC_{50} of ≈50 nM (Fig. 2*A*). The sensitivity of PtdIns 4-kinase to inhibition by WT was about one order of magnitude less than that of the PtdIns 3-kinase (IC_{50}: 3–5 nM) which was immunoprecipitated from adrenal glomerulosa cells with anti-p85 antibody (Fig. 2*B*). The activity of the soluble PtdIns kinase was increased by Triton X-100 (which inhibits PtdIns 3-kinases), and its phosphorylation product was identified as PtdIns(4)*P* (Fig. 2*B Inset*). When the partially purified PtdIns 4-kinase was resolved on a Superose-12 size exclusion column (preequilibrated with buffer A/0.5 M NaCl), the enzyme activity eluted as a peak at ≈125 kDa (data not shown).

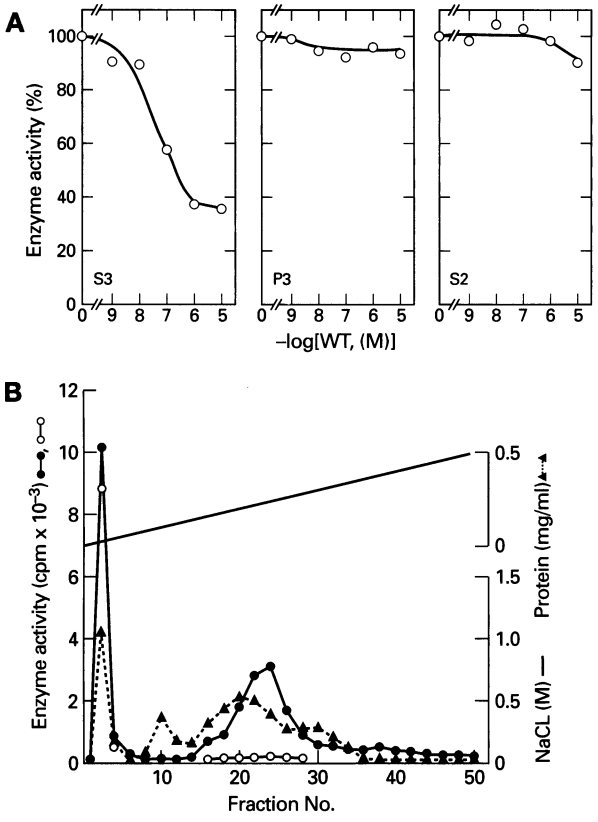

FIG. 1. Effects of WT on the activities of PtdIns 4-kinases and PtdIns(4)*P* 5-kinase obtained from adrenal cortical tissues (*A*) and separation of WT-sensitive and -insensitive soluble PtdIns 4-kinase activity by DEAE-Sepharose column chromatography (*B*). Adrenocortical homogenates were fractionated and their PtdIns 4-kinase and PtdIns(4)*P* 5-kinase activities were measured as described. The S1, S2, and S3 fractions represent the respective supernatants obtained after centrifuging the homogenates and the subsequent low-salt and high-salt wash, respectively, and P3 is the activity remaining in the membrane pellet after these washes. The specific activities of PtdIns 4-kinase in S3 and P3 fractions and that of PtdIns(4)*P* 5-kinase in the S2 fraction were 206 ± 8, 578 ± 30, and 58.9 ± 3.1 pmol/min per mg, respectively. The $(NH_4)_2SO_4$ precipitate of the S3 fraction was applied to a DEAE–Sepharose fast-flow column, washed with 7.5 ml of buffer A, and eluted with a linear gradient of 0–0.5 M NaCl in buffer A. PtdIns 4-kinase activity of each fraction was measured in the absence (●) and presence (○) of 10 μM WT. The specific activity of the WT-sensitive PtdIns 4-kinase in this preparation was 322 ± 53.6 pmol/min per mg.

Experiments on intact adrenal cells indicated that a WT-sensitive enzyme contributes to the maintenance of cellular inositolphospholipid pools during agonist stimulation. In primary cultures of adrenal glomerulosa cells labeled with *o*-[^{32}P]phosphate, 10-min pretreatment with 10 μM WT caused a marked decrease in labeled PtdIns(4)*P* but only a slight reduction in PtdIns(4,5)P_2 (Fig. 3*A*). Furthermore, stimulation of such WT-pretreated cells with the Ca^{2+}-mobilizing hormone, Ang-II, caused a rapid and sustained loss of ≈80% of the labeled PtdIns(4,5)P_2 pool instead of the transient decrease observed in Ang-II-stimulated control cells (Fig. 3*B*). The very low PtdIns(4)*P* levels of WT-treated cells remained unchanged throughout Ang-II stimulation, in contrast to the higher basal levels and the transient decrease observed during stimulation of control cells (Fig. 3*B*). Consistent with the inability of WT-treated glomerulosa cells to replenish their inositolphospholipid pools during agonist stimulation, Ang-II caused only a brief increase in Ins(1,4,5)P_3 levels instead of the prolonged and biphasic elevation observed in control Ang-II-stimulated cells (Fig. 3*D* and ref. 27). In accord with this, Ang-II caused only a transient increase in $[Ca^{2+}]_i$ in WT-pretreated cells, without the sustained plateau seen in control cells (Fig. 3*C* and ref. 27).

The effects of WT were further examined in other cell types in which ligand activation causes activation of tyrosine kinases and phospholipase Cγ, as opposed to the G protein-mediated activation phospholipase Cβ that mediates the response to Ang-II stimulation. Human Jurkat T-cell lymphoblasts stimulated by anti-CD3 antibody and NIH 3T3 cells stimulated by

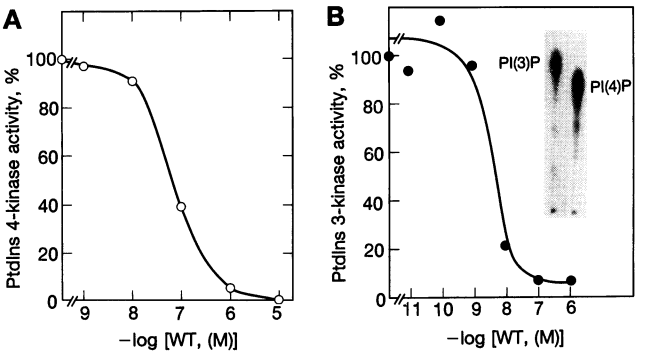

FIG. 2. WT sensitivity of the soluble adrenal PtdIns 4-kinase (*A*) and the bovine adrenal PtdIns 3-kinase immunoprecipitated from cultured adrenal glomerulosa cells with anti-p85 antibody (*B*). PtdIns 4-kinase eluted from the DEAE–Sepharose column and the PtdIns 3-kinase present in the immunoprecipitate were assayed with [γ-^{32}P]ATP after a 10-min preincubation with the indicated concentrations of WT. The reaction product was separated by TLC and quantified in a PhosphorImager. The identity of the PtdIns*P* product of the PtdIns kinase assays was assessed by resolving the ^{32}P-labeled products in a TLC system that separates PtdIns(3)*P* [PI(3)P] and PtdIns(4)*P* [PI(4)P] (26) (*B Inset*).

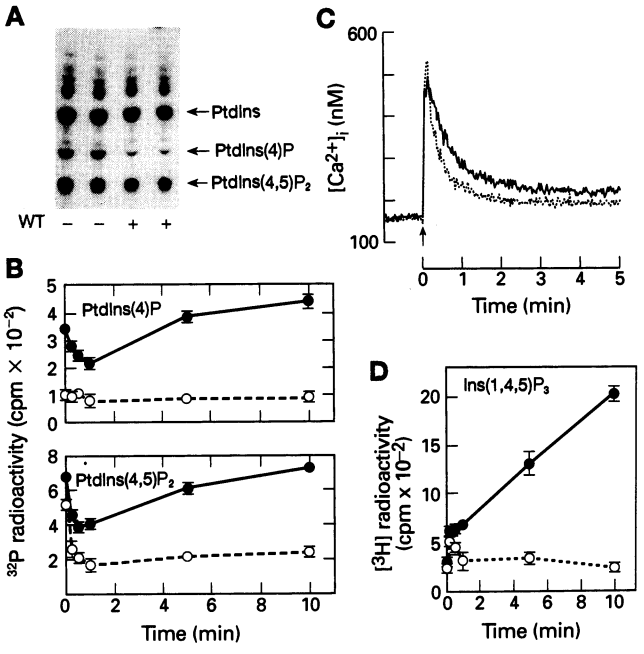

FIG. 3. Effect of WT (10 μM) on ^{32}P-labeled phospholipids (*A* and *B*), cytoplasmic [Ca^{2+}] (*C*), and Ins(1,4,5)P_3 (*D*) in adrenal glomerulosa cells. (*A*) Cultured glomerulosa cells were labeled with [^{32}P]phosphate (at 2 μCi/ml) for 4 hr. Cells were then preincubated with or without 10 μM WT for 10 min, and after an acidic extraction, inositolphospholipids were separated by TLC. Duplicate determinations from control (*Left*) and WT-treated (*Right*) cells. Positions of standard inositolphospholipids are indicated at right. (*B*) Time course of changes in the ^{32}P-labeled PtdIns(4)*P* and PtdIns(4,5)P_2 levels in Ang-II-stimulated adrenal glomerulosa cells. *o*-[^{32}P]phosphate-labeled cells were preincubated with (○) or without (●) 10 μM WT for 10 min and subsequently stimulated with 30 nM Ang-II for the times indicated. Results are given as the means ± range from duplicate determinations from a representative of three similar observations. [Ca^{2+}]$_i$ (*C*) and Ins(1,4,5)P_3 (*D*) responses in adrenal glomerulosa cells stimulated by 30 nM Ang-II were measured in fura-2-loaded and *myo*-[^{3}H]inositol-labeled glomerulosa cells, respectively. Cells were preincubated with (···) or without (—) 10 μM WT for 10 min, before stimulation with Ang-II for the indicated times. The levels of Ins(1,4,5)P_3 were measured in cells prelabeled with *myo*-[^{3}H]inositol at 40 μCi/ml for 24 hr. Due to the compressed time scale, the rapid initial Ins(1,4,5)P_3 peak cannot be distinguished from the secondary steady increase (see also ref. 27). Representative Ca^{2+} traces from six experiments and means ± range of duplicate determinations from one of five similar inositol phosphate experiments are shown.

platelet-derived growth factor showed similar inhibition profiles of their [Ca^{2+}]$_i$ and Ins(1,4,5)P_3 responses by 10 μM WT—i.e., the initial increases were only minimally affected, but the sustained phases of both inositolphosphate and Ca^{2+} signaling were completely abolished in both cell types (Fig. 4 *A–C*). At concentrations below 300 nM, WT had no significant effect on either Ins(1,4,5)P_3 formation or [Ca^{2+}]$_i$ responses (data not shown), indicating that the complete inhibition of PtdIns 3-kinase that occurs at these concentrations is not responsible for its inhibitory effects on inositol phosphate production. The transient increases of Ins(1,4,5)P_3 seen in all cell types suggested that WT did not inhibit the major forms of phospholipase C activity of the intact cells, and this result was confirmed in *in vitro* assays using purified phospholipase C isozymes. [*In vitro* assays (28) of purified phospholipase C activities (β_1, γ_1, and δ_1 enzymes) revealed no inhibition by WT up to a concentration of 10 μM (Y. S. Bae, S. G. Rhee, and T. B., unpublished observations).]

We previously reported that WT inhibits the sustained phase of Ang-II-stimulated inositol phosphate- and Ca^{2+}-signaling in adrenal glomerulosa cells (27),and similar effects of micromolar concentrations of WT have been described in thrombin-stimulated platelets (29). These effects of WT can be attributed to the marked decrease in the resting PtdIns(4)*P* pool in ^{32}P-labeled glomerulosa cells and the irreversible loss of the labeled PtdIns(4,5)P_2 pool upon agonist stimulation due to impairment of its resynthesis by inhibition of the relevant PtdIns 4-kinase. The WT concentrations required for inhibition of Ang-II-stimulated Ins(1,4,5)P_3 production in the intact cells are higher than those required to inhibit the partially purified PtdIns 4-kinase, probably reflecting the different conditions (presence of Triton X-100, lower ATP concentrations, lack of membrane barriers) used for the enzyme assay.

The relationship between the soluble PtdIns 4-kinase described in the present study and the tightly membrane-bound type II and type III enzymes is unclear at present. The WT-sensitive PtdIns 4-kinase has an apparent molecular size of 125 kDa and is less sensitive to adenosine than the membrane-bound PtdIns 4-kinase activity (IC_{50} ≈2 mM and 300 μM, respectively, using the above-described assay conditions). In this regard this enzyme clearly differs from the smaller, adenosine-sensitive type II enzyme and is more similar to the type III enzyme. That the large type III enzyme activity of the membrane contains the WT-sensitive enzyme as part of a protein complex cannot be ruled out. This possibility is not unlikely because the same PtdIns 4-kinase purified from yeast cytosol has also been isolated and cloned from the yeast nuclear-pore protein complex (30). A recently cloned human PtdIns 4-kinase (31) that shares substantial sequence homology with the yeast PtdIns 4-kinase STT4 (32) is believed to be a type II PtdIns 4-kinase, whereas the mammalian homolog of the yeast-soluble, 125-kDa PtdIns 4-kinase has been predicted to be a type III enzyme (31). The characteristics of the WT-sensitive PtdIns 4-kinase suggest that it is probably a member of the latter group of enzymes.

The small proportion of the WT-sensitive PtdIns 4-kinase activity within the total PtdIns 4-kinase activity after tissue fractionation explains why PtdIns 4-kinase was not found to be sensitive to WT in most of the above-mentioned studies on PtdIns 3-kinase (33–35). However, it should be noted that the PtdIns 4-kinase assays performed in those, as well as in the present studies, used artificial conditions (exogenous PtdIns and detergents) and, therefore, may not reflect the activities present in intact cells. In fact, PtdIns 4-kinase assays in digitonin-permeabilized adrenal glomerulosa cells, in which endogenous substrate is used, revealed that 50–60% of the activity was inhibited by WT (S.N. and T.B., unpublished observations). The present results also show that the WT-insensitive PtdIns 4-kinase(s) are unable to provide the constant supply of PtdIns(4)*P* and PtdIns(4,5)P_2 that is required to maintain the Ins(1,4,5)P_3/Ca^{2+} responses of agonist-stimulated cells. The role(s) of these enzymes in the regulation of various pools of cellular inositolphospholipids remains to be clarified. It is important to note that the relatively short labeling protocol with [^{32}P]phosphate preferentially labels the agonist-sensitive polyphosphoinositide pools, a feature used in many early studies on hormonal regulation of inositolphospholipid metabolism (36, 37). However, in glomerulosa cells labeled with *myo*-[^{3}H]inositol for 24 hr, Ang-II stimulation after WT pretreatment caused only a 50% loss of the labeled PtdIns(4,5)P_2 pool, the rest being resistant to agonist stimulation. In addition, the labeled PtdIns(4)*P* pool was reduced to ≈30% of its original level after 10 min of WT treatment and remained unchanged during Ang-II stimulation (S.N. and T.B., unpublished results). This finding is consistent with the presence of additional pools of polyphosphoinositides that probably serve other functions and are synthesized by distinct enzymes in specific cellular compartments.

In summary, we have characterized the inhibitory actions of WT on a distinctive PtdIns 4-kinase activity of adrenal glomerulosa cells and adrenocortical tissue. Among the multiple

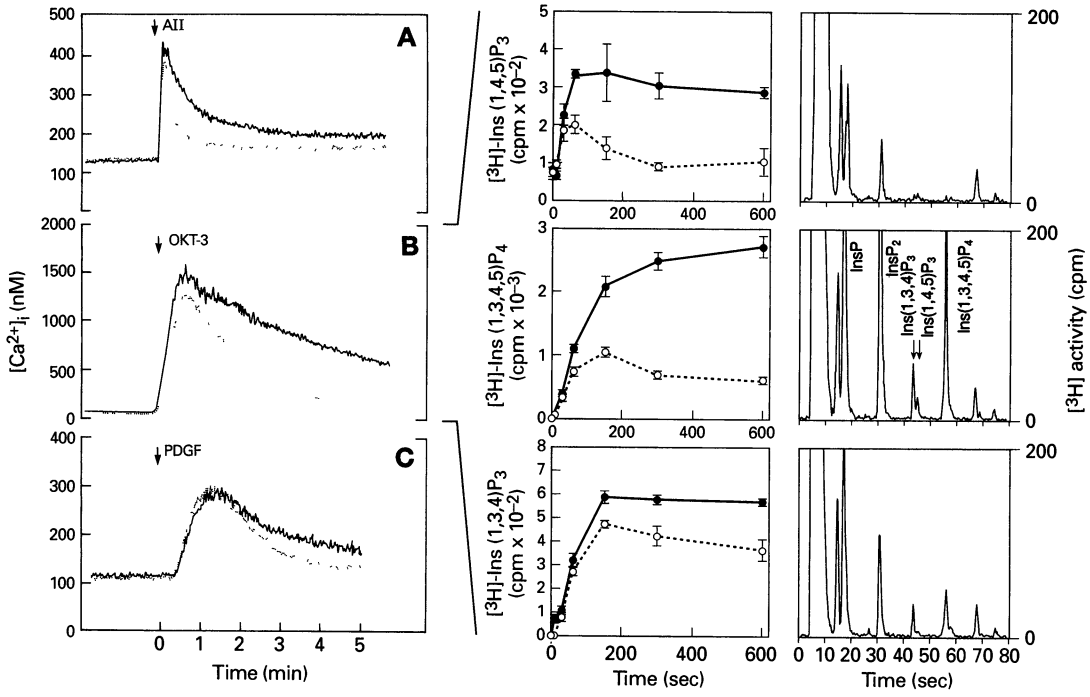

FIG. 4. Effect of WT on $[Ca^{2+}]_i$ responses in adrenal glomerulosa cells (*A*), Jurkat lymphoblasts (*B*), and NIH 3T3 cells (*C*). Cells were loaded with the fluorescence indicator fura-2 and incubated with WT (10 μM) (···) or with dimethyl sulfoxide (—) for 10 min before stimulation with 30 nM Ang-II (*A*), OKT-3 antibody at 10 μg/ml (*B*), or platelet-derived growth factor (PDGF) at 30 ng/ml (*C*). Representative traces from at least three similar observations are shown. Effects of WT on the inositol phosphate response of Jurkat lymphoblasts (*Middle* and *Right*). Cells were labeled with *myo*-[^{3}H]inositol for 24 hr and stimulated with OKT-3 antibody at 10 μg/ml for the indicated times. Representative HPLC elution profiles of the inositol phosphates from control cells and from cells stimulated with the antibody for 10 min after preincubation in the absence or presence of 10 μM WT. (*Left* Top to Bottom) Detailed time courses of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, and Ins(1,3,4)P_4 production after stimulation with OKT-3 antibody in control cells (●) or in cells preincubated with 10 μM WT (○). Means ± range of duplicate determinations are shown from one of two similar experiments (*Middle*).

forms of PtdIns 4-kinase, WT inhibits a soluble enzyme that regulates the synthesis of the hormone-sensitive pools of inositolphospholipids and participates in the sustained generation of Ins(1,4,5)P_3. The differential effects of WT on the several PtdIns 4-kinases should facilitate studies on their functions and regulation, as well as the isolation of the activity responsible for the maintenance of PtdIns(4,5)P_2 levels during agonist stimulation.

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